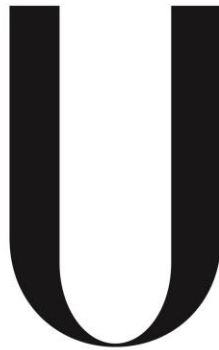


Universidade de Lisboa  
Faculdade de Ciências

Departamento de Biologia Vegetal



LISBOA

---

UNIVERSIDADE  
DE LISBOA

**The Role of SMN protein in microRNA  
biogenesis**

**Inês do Carmo Gil Gonçalves**

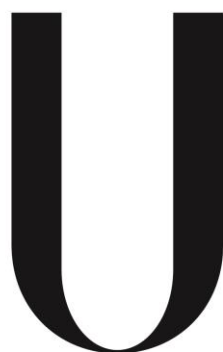
Dissertação  
Mestrado em Biologia Molecular e Genética

2013

Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal



LISBOA

---

UNIVERSIDADE  
DE LISBOA

**The Role of SMN protein in microRNA  
biogenesis**

**Inês do Carmo Gil Gonçalves**

Dissertação orientada pelo Prof. Doutor Júlio António Bargão Duarte e  
Doutora Min Jeong Kye  
Mestrado em Biologia Molecular e Genética

2013

## TABLE OF CONTENTS

<b>RESUMO EM LÍNGUA PORTUGUESA DA DISSERTAÇÃO</b>	<b>IV</b>
<b>DISSERTAÇÃO</b>	<b>1</b>
RESUMO	2
ABSTRACT	3
INTRODUCTION	4
<i>Autosomal recessive proximal spinal muscular atrophy (SMA)</i>	4
<i>Survival of motor neuron protein (SMN)</i>	
<i>and SMN complex</i>	5
<i>microRNAs (miRNAs) - biogenesis, stability and decay</i>	6
<i>microRNA in the nervous system</i>	8
<i>microRNAs and spinal muscular atrophy</i>	8
<i>Aims</i>	9
 METHODS	 10
<i>Isolation of spinal cord and brain from mice</i>	10
<i>Genotyping</i>	10
<i>RNA isolation from brain and spinal cord</i>	10
<i>cDNA synthesis and real time PCR for pri-miRNA and miRNA</i>	10
<i>Primers for genes in miRNA biogenesis and stability pathways;</i>	
<i>expression and splicing</i>	11
<i>cDNA synthesis and real time PCR for mRNA</i>	11
<i>Polymerase Chain Reaction (PCR)</i>	
<i>for splicing characterization</i>	11
<i>Gel Extraction</i>	12
<i>Protein isolation from mouse tissue</i>	12
<i>Protein quantification</i>	12
<i>Western Blot</i>	13

<i>Antibodies</i>	13
<i>Statistical Analysis</i>	13
 RESULTS	 14
<i>Quantification of pri-miRNA and miRNA expression of miR-183~96~182 cluster</i>	 14
<i>Quantitative real time-PCR analysis of genes involved in miRNA biogenesis and decay pathways</i>	 15
<i>Characterization of splicing in genes involved in miRNA biogenesis and decay pathways</i>	 16
<i>Analysis of gene expression involved in miRNA biogenesis and decay pathways in protein level</i>	 17
 DISCUSSION	 19
ACKNOWLEDGEMENTS	21
REFERENCES	22
 ANEXOS	 S1

**Resumo em Língua Portuguesa da Dissertação intitulada: “The role of SMN protein in microRNA biogenesis”**

A Atrofia Muscular Espinal (AME) é uma doença genética autossômica recessiva, caracterizada por degeneração dos neurónios alfa inferiores localizados no corno anterior da medula espinhal. Indivíduos com AME manifestam atrofia e paralisia muscular progressiva. Sendo uma das doenças mais comuns do sistema nervoso, e a principal causa hereditária de mortalidade infantil, esta doença foi classificada em 4 grupos clínicos: Tipo I (AME infantil – Werdnig-Hoffman), Tipo II (AME intermédia), Tipo III (AME juvenil – Kugelberg-Welander) e Tipo IV (AME adulta).

Uma mutação homozigótica no gene *Survival Motor Neuron-1 (SMN1)* é a causa determinante da AME. O gene *SMN1* está localizado numa dupla região invertida do cromossoma 5q11.2-13.3, onde é também possível encontrar uma cópia sua, bastante idêntica, designada *SMN2*. Uma transição C-T na posição +6 do exão 7 do gene *SMN2* interfere com o seu processo de splicing, levando à exclusão deste exão. Assim, enquanto o gene *SMN1* codifica na sua totalidade proteína *Survival of Motor Neuron* funcional (FL-SMN), o gene *SMN2* produz 90% de proteína truncada e instável (SMN $\Delta$ 7) e 10% de proteína funcional (FL-SMN), quantidade esta não suficiente para conferir protecção contra a severidade da doença. SMN é uma proteína de expressão ubíqua, com níveis de expressão particularmente elevados em órgãos como medula espinhal, cérebro e músculos. Em associação com 7 outras proteínas, denominadas Gemins (Gemin2-8), SMN forma um complexo – o complexo SMN. O complexo SMN está envolvido no processo de montagem das ribonucleoproteínas nucleares pequenas (*small-nuclear ribonucleoprotein – snRNPs*), sendo esta a sua principal função. snRNPs são um componente fundamental do spliceossoma, onde são responsáveis pelo reconhecimento da molécula de pré-RNA-mensageiro (*pre-messengerRNA – pre-mRNA*) e remoção de intrões. De igual forma, a proteína SMN desempenha um papel neuro-específico, manifestado através da interferência na expressão e transporte de RNA mensageiro (mRNA) ao longo dos axónios e interferência na síntese proteica que ocorre nas neurites dos neurónios motores.

Os microRNAs (miRNAs) são moléculas endógenas de RNA, de cadeia simples e não codificantes, com importante função na regulação da expressão génica a nível dos processos de transcrição e pós-transcrição. Após a transcrição pela enzima RNA polimerase II, é gerada uma molécula primária (pri-miRNA) em forma de *hairpin*. Esta estrutura é especificamente reconhecida e clivada pela ribonuclease Drosha e o seu co-factor, *DiGeorge syndrome critical region gene* (DGCR8), originando no núcleo uma molécula precursora (pré-miRNA) de ~70 nucleótidos. Seguidamente, esta molécula precursora é transportada pela Exportin5 para o citoplasma, onde ocorre uma segunda clivagem efectuada pela ribonuclease Dicer, que actuando juntamente com outros factores dá origem a um duplex miRNA/miRNA\* com ~20 nucleótidos. Uma das cadeias deste duplex, a chamada cadeia guia, é incorporada na proteína Argonauta2 (AGO2), enquanto a cadeia passageira será degradada. AGO2, carregada com a cadeia guia, é então incorporada no complexo RISC, onde por compatibilidade através da região 3'-*untranslated region* (3'UTR), se liga ao mRNA alvo, levando ao seu silenciamento por clivagem ou repressão do processo de tradução. A estabilidade da molécula madura de miRNA é controlada por uma série de factores de actuação *cis* e *trans*, formação de complexos proteicos e exposição a nucleases. Em animais, o decaimento de miRNA maduro é controlado pelas exoribonucleases Xrn1 e Xrn2.

Os miRNAs são moléculas expressas em elevado número no sistema nervoso, onde actuam em processos de desenvolvimento, especificação celular, modelação e plasticidade neuronal (plasticidade sináptica). Várias doenças neurológicas estão associadas a uma desregulação na expressão de miRNAs, incluindo AME. Gemin3 e 4 são duas das proteínas Gemin que, para além de constituírem o complexo SMN, estão também ligadas à proteína AGO2 e, consequentemente, ao complexo RISC. Igualmente, várias proteínas que interactuam com a proteína SMN estão envolvidas nos processos de biogénese e decaimento dos miRNAs.

Recentemente foi demonstrada a importância da actividade dos miRNAs para a sobrevivência a longo termo de neurónios motores de medula espinhal *in vivo*. Além disto, foram detectadas alterações na expressão e distribuição de vários miRNAs, destacando-se um aumento constante na expressão do microRNA-183 (miR-183) causado pela perda da proteína SMN.

Assim, uma vez que a proteína SMN actua na regulação e transporte de mRNAs para serem localmente traduzidos, foi colocada em questão uma possível interferência do complexo SMN nos processos de biogénese e decaimento dos miRNAs.

Numa tentativa de compreender qual o mecanismo subjacente à desregulação dos miRNAs causada pela perda de SMN, medimos os níveis de expressão das formas primária e madura do miR-183. Sendo que este miRNA é transcrito de forma única a partir do *cluster* miR-183~96~182, também a expressão das formas primária e madura dos miR-96 e miR-182 foi medida em medula espinhal isolada a partir de um modelo animal de AME, o qual inclui 2 cópias por alelo do gene *SMN2* humano (FVB; P4, *Smn*<sup>-/-</sup>; *SMN2*<sup>tg0</sup>). Os resultados obtidos através de real-time PCR (RT-PCR) mostraram não haver alterações na quantidade de transcritos primários dos 3 miRNAs que formam o *cluster*, enquanto a forma madura do miR-183 e miR-96 apresentaram valores de expressão significativamente elevados. No entanto, miR-182 não revelou qualquer alteração nos seus níveis de expressão. Assim, estes resultados sugerem que existe uma regulação diferencial na biogénese dos miRNAs constituintes do *cluster* miR-183~96~182, e da mesma forma, confirmam que a perda de SMN leva a uma desregulação na biogénese dos miRNAs.

Para perceber o mecanismo molecular através do qual a proteína SMN afecta a expressão dos miRNAs, caracterizámos o processo de biogénese destas moléculas, desde a sua transcrição primária até todos os passos seguintes de processamento de RNA. Para tal, através de RT-PCR quantificámos a expressão de transcritos dos genes envolvidos na biogénese e decaimento dos miRNAs em medula espinhal de um modelo animal de AME. Curiosamente observámos uma diminuição na expressão de todos os genes em estudo, com excepção de Drosha e Exportin5, os quais não apresentaram alterações significativas nos seus níveis normais de expressão. Consequentemente, esta regulação negativa observada demonstra que a proteína SMN desempenha um papel na desregulação dos genes envolvidos na biogénese e estabilidade dos miRNAs.

Tendo em conta o papel importante da proteína SMN no processo de splicing, analisámos este processo nos genes envolvidos na biogénese e decaimento dos miRNAs. Especificamente

pretendemos verificar se a perda de SMN provoca alguma alteração no processo normal de splicing dos genes em estudo, levando à ruptura de algum dos transcritos já conhecidos, ou criando um novo variante funcional ou não funcional. Assim, acedemos à plataforma EURASNET, a partir de onde recolhemos toda a informação disponível acerca do splicing alternativo e transcritos existentes para os genes em estudo. Consultando a estrutura genómica destes genes, escolhemos a região mais propícia a sofrer splicing, desenhando pares de primers específicos para os exões que flanqueiam a região determinada. Curiosamente, os resultados observados revelaram que o processo de splicing não sofre alterações devido à redução de SMN. Para confirmar este pressuposto, os fragmentos amplificados por PCR foram enviados para sequenciação. No entanto, apesar de não encontrarmos alterações no processo de splicing, foi detectada uma redução na expressão dos fragmentos amplificados nas amostras de SMA quando comparadas com os controlos. Redução que, através de análise estatística, confirmámos ser significativa para os genes Ago1, Exportin5 e Xrn2.

Depois de demonstrar uma regulação negativa dos genes envolvidos na biogénese e decaimento dos miRNAs, confirmámos se o mesmo se verifica a nível proteico. Para tal, através da realização de Western Blot, medimos a expressão proteica dos genes em estudos. Semelhante ao verificado para a expressão a nível dos transcritos, genes como Dgcr8, Ago1 e Xrn1 apresentaram uma redução significativa nos seus valores normais de expressão. De igual modo, foi também verificada uma pequena redução na expressão dos restantes genes testados, com única excepção da Drosha.

Em conclusão, este estudo demonstra que existe uma desregulação na expressão do *cluster* miR-183~96~182 no modelo animal de AME, sendo provável que esta ocorra a nível pós-transcricional, uma vez que a transcrição do agregado não apresentou alterações. Da mesma forma, observámos que a perda da proteína SMN interfere na expressão dos miRNAs através de uma desregulação dos genes envolvidos na biogénese, estabilidade e decaimento dos miRNAs. Assim, este estudo contribui para o enriquecimento do conhecimento acerca da relação mecanística entre a proteína SMN e os miRNAs, oferecendo informação adicional acerca do papel que os miRNAs desempenham na patologia AME.



# The role of SMN protein in microRNA biogenesis



**INSTITUTE OF  
HUMAN GENETICS**  
UNIVERSITY OF COLOGNE

Inês do Carmo Gil Gonçalves

Supervised by:

Min Jeong Kye, Ph.D

Júlio António Bargão Duarte, Ph.D

M. Sc. in Molecular Biology and Genetics

2013

## RESUMO

A Atrofia Muscular Espinal (AME) é uma doença neuromuscular caracterizada pela degeneração dos neurónios alfa motores inferiores, atrofia muscular e perda de função motora. AME é causada por uma mutação homozigótica ou deleção no gene *Survival Motor Neuron-1* (*SMN1*). No entanto, é desconhecida ainda a razão pela qual a perda de função da proteína SMN afecta especificamente os neurónios motores inferiores. Os microRNAs (miRNA) são uma categoria de RNA não codificante expressos em grande quantidade no sistema nervoso. Foi demonstrado que desregulações na expressão dos miRNAs estão associadas a algumas doenças neurológicas, incluindo AME. Especificamente, detectámos um aumento na expressão do microRNA-183 (miR-183) em neurónios com expressão nula da proteína SMN. Desta forma, como objectivo de estudo pretendemos descobrir quais os mecanismos que estão envolvidos na desregulação da expressão dos microRNAs causada pela perda de SMN. Uma vez estando o complexo SMN envolvido no processamento de RNA, foi posto em questão um possível envolvimento deste complexo na regulação da expressão dos microRNAs. Para testar esta hipótese, analisámos o processo de biogénese dos miRNAs desde a sua transcrição primária até aos restantes passos do processamento de RNA, em medula espinal de um modelo animal de AME. A análise de expressão feita ao *cluster* miR-183~96~182 mostrou uma desregulação na regulação do processo de biogénese deste *cluster* em neurónios onde a proteína SMN não é expressa. Da mesma forma, verificámos uma desregulação na expressão dos genes envolvidos na biogénese, estabilidade e decaimento dos miRNAs em medula espinal de um modelo animal de AME. Assim, os resultados obtidos confirmam que a expressão dos miRNAs é alterada devido a níveis deficientes da proteína SMN.

**Palavras-chave:** Proteína *Survival motor neuron* (SMN), microRNA, *pathways* de biogénese e estabilidade, expressão, desregulação

**ABSTRACT**

Spinal muscular atrophy (SMA) is a neuromuscular disease caused by degeneration of alpha lower motor neurons, muscle atrophy and loss of motor function. SMA occurs when *SMN1* (*survival motor neuron-1*) is homozygously mutated or deleted. However, it remains unclear how deficiency of SMN function mainly affects lower motor neurons. microRNA (miRNA) are a subset of non-coding RNAs highly expressed in the nervous system. It has been reported that dysregulation of miRNA expression is associated to several neurological diseases including SMA. We found that expression of miR-183 is elevated in SMN deficient neurons. Therefore, we sought to uncover the mechanisms underlying the dysregulation of miRNA expression caused by SMN loss. Since the SMN complex plays a role in RNA processing, we hypothesize that this complex is involved in regulation of miRNA expression. To test our hypothesis, we analyzed miRNA biogenesis from primary transcript to down-stream steps of RNA processing in spinal cord of SMA mouse model. Analysis of the expression of miR-183~96~182 cluster suggests that the biogenesis process for this cluster is dysregulated in SMN deficient neurons. We found that the genes involved in miRNA biogenesis, stability and decay pathways are dysregulated in spinal cord of SMA mouse model. Our findings suggest that SMN deficiency causes dysregulation of miRNA biogenesis and decay pathways.

**Keywords:** Survival of motor neuron protein (SMN), microRNA (miRNA), biogenesis and decay pathways, expression, dysregulation

## INTRODUCTION

### **Autosomal recessive proximal spinal muscular atrophy (SMA)**

Spinal muscular atrophies (SMAs) are a genetically and clinically heterogeneous group of neuromuscular disorders characterized by progressive degeneration of lower alpha motor neurons in the anterior horns of the spinal cord.<sup>1</sup> Affected individuals exhibit autosomal recessive inheritance with proximal manifestation of muscle weakness and atrophy defined as autosomal recessive proximal spinal muscular atrophy (SMA).<sup>2</sup> With an incidence of about 1:6000 to 1:10000 newborns and a carrier frequency of 1:35, SMA is the leading hereditary cause of infant mortality.<sup>3,4</sup> Due to the highly variable disease severity, four clinical types of SMA are classified based on the age of onset and achieved motor abilities: Type I SMA (Werdnig–Hoffmann), intermediate Type II SMA, mild Type III SMA (Kugelberg–Welander) and Type IV SMA (adult SMA).<sup>2,4-8</sup>

*Survival of motor neuron-1 (SMN1)* is the disease-determining gene of SMA, leading to the disease due to a homozygous deletion or mutation.<sup>9</sup> This gene is located on the chromosomal region 5q11.2-13.3 in a segment of ~500kb, which includes the telomeric *SMN1* and the similar but slightly different centromeric *SMN2*.<sup>10</sup> The human genome contains a copy of *SMN1* and several copy numbers of *SMN2* genes.<sup>11</sup> With 99% of identity, the two SMN copies only differ in 5 base-pair exchange localized within the 3' end of the genes.<sup>9,12</sup> However, only the C-to-T transition at position +6 of exon 7 (c.840C>T) is localized within the coding region.<sup>13</sup> Although it is a translational silent mutation, it severely affects the correct splicing of the exon 7 by disrupting an exonic splicing enhancer (ESE) and generating an exonic splicing silencer (ESS) instead. Therefore, an alternative spliced mRNA isoform that lacks this exon (*SMNΔ7*) is produced.<sup>12,14</sup> *SMN1*-derived transcripts produce full-length (FL) and functional SMN protein, while nearly 90% of *SMN2*-derived transcripts generate a truncated and instable protein (*SMN2Δ7*). Although low amount of FL-*SMN2* is also produced, which is equivalent to ~10% of *SMN1* levels, it is not enough to protect against disease development.<sup>2,15</sup> Since the clinical severity of SMA depends on the amount of SMN protein, the copy numbers of *SMN2* directly impacts disease severity.<sup>16-18</sup>

### Survival of motor neuron protein (SMN) and SMN complex

The human SMN protein is a 38kDa protein with 294 amino acids, encoded by 8 exons.<sup>9</sup> Is a ubiquitously expressed protein, with particularly higher levels in spinal cord, brain, kidney and liver. However, in individuals with Type I SMA was detected a 100-fold reduction of SMN levels in spinal cord when compared with the controls.<sup>19</sup> Complete loss of SMN protein in animals causes embryonic lethality, pointing out the crucial role of this protein in early development.<sup>20</sup> The SMN protein is localized in both the cytoplasm and the nucleus. With a disperse distribution in the cytoplasm, the nuclear SMN is found in structures called Gems, for “*Gemini of the coiled bodies*”, which are intimately associated with the nuclear coiled bodies (Cajal bodies).<sup>19,21</sup>

SMN is tightly associated with its binding partners and forms the SMN complex. These proteins are named Gemin (Gemin 2-8) due to their localization in nuclear structures, Gems. SMN self-oligomerization creates the backbone of the complex.<sup>23,24</sup> Gemin2, Gemin3, Gemin5 and Gemin7 directly interact with SMN, whereas Gemin4 and Gemin6 are connected with the complex through their interactions with Gemin3 and Gemin7, respectively. The direct binding of Gemin8 with the SMN complex mediates its interaction with the Gemin6/Gemin7 heterodimer, which binds directly to UNR- interaction protein (Unrip).<sup>23-25</sup> The most well characterized function of the SMN complex is the ATP-dependent assembly of the heptameric core of Sm proteins on Uridine-rich snRNAs (U-snRNAs), which are involved in the formation of the spliceosomal small nuclear ribonucleoproteins (snRNPs). SnRNPs are crucial factors within the spliceosome, playing an important role in the recognition and splicing of introns of pre-mRNA in the nucleus.<sup>11,23,26,27</sup> An snRNP molecule consists in one U-snRNAs molecule, a common core comprising a ring of seven common Sm proteins, and several snRNP-specific proteins.<sup>11,23,26</sup> SMA animal models show a reduced U-snRNP assembly activity in the central nervous system, as well as signals of motor axon degeneration due to impaired U-snRNPs assembly.<sup>7,27</sup>

Immunocytochemical studies described an association between SMN and cytoskeletal elements in the axons and dendrites of neurons.<sup>28,29</sup> Endogenous SMN and Gemin proteins co-localized in granules which exhibit rapid, active and bidirectional movement that extends throughout neurites and growth cones of cultured motor neurons.<sup>30</sup> Local translation is known to be implicated in

SMA pathology. The SMN binding partner hnRNP is associated with the 3' UTR of  $\beta$ -actin mRNAs, a well-known mRNA that undergoes local translation. SMN deficient motor neurons show alterations of  $\beta$ -actin protein and mRNA localization in axons and growth cones of developing neurons, which might explain the deficit in axonal actin cytoskeleton organization due to low SMN levels.<sup>23,31</sup> Additionally, it was shown that SMN forms a complex with HuD that binds to the neuritin (cpg15) mRNA and is involved in the transport and deliver of mRNA in motor neuron axons to be locally translated.<sup>32</sup> Thus, it is suggested that SMN plays a neuronal-specific role in axonal mRNA expression/trafficking and local protein synthesis in the neurites<sup>21,32,33</sup> Likewise, it has been shown a neuronal specific role of SMN in axonal outgrowth and pathfinding.<sup>34,35</sup> Low SMN levels cause alteration in neuromuscular junctions (NMJ) morphology.<sup>36,37</sup> SMN binds to others RNA binding proteins as *Drosophila* homologue of the X mental retardation protein (FMRP), KH-type splicing regulatory protein (KSRP) and FUS/TLS.<sup>38-40</sup>

### **microRNAs (miRNAs) - biogenesis, stability and decay**

microRNAs (miRNAs) are endogenous non-coding single-stranded RNAs (ssRNA) molecules of ~22 nucleotides that play important gene-regulatory roles in animals and plants through transcriptional and posttranscriptional regulation. miRNAs function via base-pairing to the 3'-untranslated region (3'-UTR) of messenger RNAs (mRNAs) from target protein-coding genes, leading to gene silencing by mRNA cleavage, translational repression and deadenylation.<sup>41</sup> Functional studies indicate that miRNAs participate in the regulation of a wide range of cellular cellular and developmental processes. Dysregulations of specific miRNAs related pathways are associated with several human pathologies.<sup>42,43</sup>

The majority of characterized miRNAs are transcribed by RNA polymerase II from independent genes or introns of protein-coding genes resulting in primary transcripts (pri-miRNAs), which fold into a hairpin structure.<sup>42</sup> This structure is specifically recognized by the nuclear ribonucleaseIII (RNaseIII) enzyme Drosha, which together with its cofactor DiGeorge syndrome critical region gene 8 (DGCR8) comprises the microprocessor complex. DGCR8 is a double stranded RNA-binding domain protein that acts as a molecular anchor necessary for the recognition of pri-miRNA. Within this complex, Drosha cleaves the pri-miRNA into a ~70-

nucleotide precursor hairpin (pre-miRNA).<sup>44</sup> Afterwards, this precursor is translocated to the cytoplasm by exportin5 (XPO5) through the nuclear pore complex in a Ran guanosine triphosphate (RanGTP)-dependent process.<sup>45</sup> Once in the cytoplasm, the pre-miRNAs are cleaved by a second complex comprising Dicer (RNaseIII enzyme) in association with the transactivation-responsive RNA binding protein (TRPB), and the protein activator of the interferon-induced protein kinase (PACT).<sup>46,47</sup> This complex processes the pre-miRNA, generating a ~20bp miRNA/miRNA\* duplex. One strand of this duplex is incorporated into the Argonaute2/ Eukaryotic Translation Initiation Factor 2C (Ago2/Eif2c2) protein as a mature miRNA (guide strand/miRNA), whereas the other strand (passenger strand/miRNA\*) is degraded.<sup>42</sup>

Ago2/Eif2c2 is loaded with miRNAs through a specialized assembly called the RISC-loading complex (RLC), which comprises the proteins Ago2, Dicer, TRBP, PACT. Therefore, is induced the assembly of a ribonucleoprotein effector complex known as the RNA-induced silencing complex (RISC), which is responsible for the silencing of target genes.<sup>48</sup> The functional core of RISC is mainly composed by members of the Argonaute family (PIWI1-4 and EIFC2C/AGO1-4 subfamilies) and glycine-tryptophan protein of 182kDa (GW182). However, only Ago2/Eif2c2 shows intrinsic endonuclease enzymatic activity, being responsible for the mRNA slicer activity in RISC. GW182 proteins act as downstream effectors of repression.<sup>42,43,49,50</sup> Ago2 has an additional independent function in miRNA biogenesis: it generates an intermediate miRNA precursor named Ago2-cleaved precursor (ac-pre-miRNA).<sup>51</sup> Additional proteins associated with RISC have been newly identified such as FMRP; members of the family of DExD/H RNA helicases, as MOV10 and RNA helicase A; R2D2; RNA-binding proteins as HuR; and Gemin3 and 4, which are thought to also have helicase activity.<sup>52</sup>

Transcription, processing and decay of miRNAs are subject to sophisticated control. The multiple steps in miRNA biogenesis seem to be remarkably well coordinated, with the transcription being the major level of control responsible for tissue and development-specific expression of miRNA. Such regulation is carried out by several activators or repressors that interact either with Dicer or Drosha, or binding to pre-miRNA.<sup>42,49,52,53</sup> The stability of mature miRNA is controlled by *cis*- and *trans*-acting modifications, protein complex formation and exposure to nucleases. AGO

proteins also play an important role in miRNA stabilization. miRNA decay is carried out by exonucleases named small RNA degrading nucleases (SDS) that catalyze 3'-to-5' decay in plants, whereas in animals such function is performed by the 5'-to-3' exoribonucleases 1 and 2 (XRN1 and XRN2).<sup>53,54</sup>

### **microRNA in the nervous system**

microRNAs are abundantly expressed in the nervous system, where its function affects a large number of neuronal genes. Changing levels of certain ubiquitous and brain-specific miRNAs shape the development and function of the nervous system. miRNAs are mainly involved in developmental process as cell specification, patterning and neuronal plasticity.<sup>55</sup> The involvement in the specification of cell types and maintenance of cell identity was additionally identified as miRNA function.<sup>55</sup> Being expressed in dendrites and axons, miRNA are thought to be involved in the control of synaptic plasticity and axonal pathfinding.<sup>55</sup> Many miRNAs can be detected in neurites of primary hippocampal and sympathetic neurons, which suggests an involvement in the control of local mRNA translation in neurons.<sup>55,56</sup> miRNA-mediated regulation seems particularly well suited in local translational control of synaptic plasticity.<sup>57,58</sup>

### **microRNAs and spinal muscular atrophy**

Among the Gemin proteins that constitute the SMN complex, Gemin3 and 4 are also binding-partners of AGO2/EIF2C2.<sup>59</sup> Several miRNAs are reported as binding to Gemin3 in human and murine neuronal cell lines.<sup>60</sup> SMN binding partners as FMRP, FUS/TLS and KSPR are also involved in miRNA biogenesis and function.<sup>38-40</sup> Therefore, it is postulated that SMN protein plays a role in miRNA expression and distribution in neurons to regulate local translation. Recent report shows that miRNA activity is essential for long-term survival of postmitotic spinal motor neuron *in vivo*. In fact, ablation of Dicer leads to a loss of ability to make functional miRNA, leading to manifestation of hallmarks characteristic of SMA. Additionally, embryonic stem cell derived motor neuron from a SMA mouse model show a specific down-regulation of miR-9 and miR9\* expression.<sup>61</sup> Such findings suggest miRNA dysregulation due to loss of SMN protein. However, cellular mechanisms involved in SMN mediated miRNA expression and functions are still unknown.



## Aims

Since SMN plays a role in the regulation of expression and/or trafficking of mRNAs that are locally translated, was hypothesized that this complex is involved in miRNA biogenesis and stability. To test this hypothesis, SMN expression was knocked down in neurons and the expression of 187 miRNAs was measured.<sup>56</sup> Previous studies and preliminary data showed that that several miRNAs exhibit changes in their distribution and expression due to SMN deficiency. The most constantly altered miRNA was miR-183; an increase in overall expression of miR-183 was reported in different cells and tissues such as embryonic cortical neurons, spinal motor neurons from SMA animal models and fibroblast cell lines derived from SMA patients (unpublished data).<sup>56</sup> miR-183 is transcribed from a miRNA cluster miR-183~96~182.

The cellular mechanism behind the relationship between the SMN protein and miRNAs is not yet identified. Therefore, the aim of this work is to identify how miRNA expression is dysregulated in SMN deficient neurons: at the transcriptional level, in the biogenesis processes or at stability and decay levels. With this study we (1) measured the expression of primary and mature transcripts from the miR-183~96~182 cluster in spinal cord of 4-days old Taiwanese SMA mice model, which contains 2 copies of the human *SMN2* per allele (FVB, P4, *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>)<sup>62</sup>; (2) measured the expression of major genes involved in miRNA biogenesis, specifically *Drosha*, *Dgcr8*, *Dicer*, *Ago1/Eif2c1*, *Ago2/Eif2c2*, *Xrn1*, *Xrn2* and *Exportin5/Xpo5* in spinal cord of the previously described SMA mouse model; (3) performed splicing analysis to test whether this process is dysregulated in miRNA biogenesis genes; and (4) analyzed the expression of the biogenesis and decay genes at the protein level, performing western blot to measure the protein expression of miRNA biogenesis from spinal cord of the SMA mouse model (FVB, P4, *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>).

## METHODS

### Isolation of spinal cord and brain from mice

Spinal cord and brain were isolated from the Taiwanese SMA mouse model (FVB; P4; *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>), produced in laboratory of Prof. Dr. Brunhilde Wirth.<sup>62</sup> Both homozygous (FVB; *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>) and heterozygous (FVB; *Smn*<sup>-/+</sup>; *SMN2*<sup>tg/0</sup>) contain 2 copies of human *SMN2* in their genome. The strain FVB/NJ was used as wild-type.<sup>62</sup> 4 days old mice were euthanized by carbon dioxide (CO<sub>2</sub>) in a closed chamber and, the spinal cord was isolated and stored at -20°C.

### Genotyping

For each animal used in this study, the genotype was checked using the Kapa<sup>TM</sup> Mouse Genotyping Hot Start Kit (Peqlab Biotechnologies, PB), according to manufacturer's protocol. A polymerase chain reaction (PCR) was performed using specific primer pairs to distinguish the homozygous from the heterozygous (oligo 5'-ataacaccaccactcttactc-3', oligo 5'-gtagccgtgatgccattgtca-3' and oligo 5'-agcctgaagaacgagatcagc-3') and to confirm the transgene (oligo 5'-cgaatcacttgagggcaggagttt-3' and oligo 5'-aactgggtggacatggctgttcattg-3'). The PCR reaction was performed with the conditions recommended by the manufacturer. The PCR products were separated in 1% agarose gel electrophoresis and visualized using ethidium bromide (ApplyChem). Images were taken with ChemiDoc XRS Imaging system (*Biorad*).

### RNA isolation from brain and spinal cord

Total RNA was extracted from spinal cord using the *mirVana*<sup>TM</sup> miRNA Isolation Kit (Applied Biosystems, AB) according to manufacturer's instructions. Total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (PB). The samples were immediately used, or stored at -20°C for future use.

### cDNA synthesis and real-time PCR for pri-miRNA and miRNA

Real time PCR was performed to measure expression levels of individual primary and mature miRNA forms of miR-182, miR-183 and miR-96. 50ng of total RNA was reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (AB). Pri-miRNA and miRNA

expression was measured by TaqMan<sup>®</sup> RT-PCR following the same procedure as described before.<sup>63</sup> The RT-PCR was performed with following conditions: an initial incubation stage at 50°C for 2min, denaturation at 95°C for 10 min and 40 cycles of detection (annealing/extension at 95°C for 15s and 60°C for 1min). All samples were analyzed in duplicated. Amplified signals were collected by 7500 System (ABI) and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### **Primers for genes in miRNA biogenesis and stability pathways; expression and splicing**

Primer pairs for genes in miRNA pathway were designed using Primer3Plus software and purchased from Metabion. The sequence of the each gene was obtained from NCBI nucleotide data-base. To check splicing variants, the genomic sequences were obtained from USCS genome browser and primer pairs were designed for the exons considered as likely to undergo splicing. Known information about splicing process in these genes was accessed through EURASNET - an alternative splicing network database.

### **cDNA synthesis and real time PCR for mRNA**

100ng of total RNA was reversed transcribed using the High Capacity cDNA Reverse Transcription Kit with random primers (AB). PowerSYBR<sup>®</sup> Green PCR Master Mix (AB) was used to amplify signals with 20ng of cDNA and 1μM of gene specific primers (**Supplementary Table1**). RT-PCR amplification conditions were: an initial incubation stage at 50°C for 2min, denaturation at 95°C for 10 min and 40 cycles of detection (95°C for 15s, 60°C for 30s, and 72°C for 40s). A final dissociation was performed to check if the amplification was correctly performed. All samples were analyzed at least twice. The RT-PCR was performed using 7500 Real Time PCR System (AB). GAPDH was used as internal control.

### **Polymerase Chain Reaction (PCR) for splicing characterization**

To characterize splicing process in the genes involved in miRNA biogenesis and stability, a PCR was carried out with 20ng/μL of cDNA isolated from spinal cord, and 1μM of each gene specific primer pair (**Supplementary Table2**). The amplification was performed with the enzyme *Taq* DNA Polymerase, Recombinant (Invitrogen) and the cycling conditions followed the

manufacturer instruction: an initial denaturation step of 3 min at 94°C, followed by 43 cycles of 45s at 94°C, gene specific primer annealing temperature (**Supplementary Table2**) for 30s, and extension at 72°C for 3min; followed by a final extension step of 10min at 72°C (Thermocycler: DNA engine Tetrad2 from Mj Research). PCR products were separated in 1% agarose gel electrophoresis and visualized with ethidium bromide (ApplyChem). Images were taken using ChemiDoc XRS Imaging system (*Biorad*).

### **Gel Extraction**

The DNA fragments amplified by PCR were excised from the agarose gel and extracted using the QIAquick Gel Extraction Kit (QIAGEN), following the manufacturer's protocol. DNA concentration was quantified with the NanoDrop ND-1000 spectrophotometer (PB), and sequence was confirmed by sequencing (GATC Biotech). Sequencing results were analyzed using SeqMan Software (Lasergene®).

### **Protein isolation from mouse tissue**

Mouse spinal cord was lysed in 400µL of Lysis Buffer (1M Tris-HCL, Triton X-100, 5M NaCl, 100mM EDTA and ddH<sub>2</sub>O) containing protease inhibitor (in a 1:24 dilution). Homogenization was carried out using the T10 basic ULTRA-TURRAX homogenizer. Hereafter, the proteins were sonicated for 10min with the Bioruptor® Plus (Diagenode), and centrifuged (5415 R centrifuge, Eppendorf) for 15min at 13.000rpm and 4°C. The protein-containing supernatant was collected for further analysis and stored at -20°C.

### **Protein quantification**

Pierce™ BCA protein assay kit (Thermo Scientific) was used to determine the protein concentration and ensure equal sample loading. We followed manufacturer's instructions for this procedure. The standard curve and concentration values were acquired using the NanoDrop ND-1000 spectrophotometer (PB).

## Western Blot

Protein expressions were analyzed by western blots.<sup>64</sup> Proteins were separated in 8% and 10% SDS gel electrophoresis and, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, pore size 0.45µm) equilibrated with Methanol. Ponceu staining (15s) was carried out to check protein transfer. The membranes were incubated in 5% milk solution for 1h at room temperature to reduce non-specific background bindings (blocking). Subsequently, the membrane was incubated with primary antibody, which the optimum incubation time and concentration were determined empirically: incubation in 5% milk overnight at 4°C. Before and after the secondary antibody incubation, the membrane was washed 15min in tris buffered saline with tween (TBS-T) buffer for 3 times. The membrane was incubated with secondary antibody for 2h at 4°C. SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) was used to detected chemiluminescence signals. The signals were obtained and analyzed by the ChemiDOC XRS (Biorad) analysis software.

## Antibodies

The following primary antibodies were used: goat anti-Drosha (ab58589, Abcam), rabbit anti-Dicer (SAB4200087, Sigma-Aldrich), rabbit anti-XRN1 (SAB4200028, Sigma-Aldrich), rabbit anti- XRN2 (SAB4500893, Sigma-Aldrich), rabbit anti-Exportin5 (SAB4200003, Sigma-Aldrich), rabbit anti-DGCR8 (SAB4200089, Sigma-Aldrich), rabbit anti-Argonaute (C34C6, Cell Signaling) and rabbit anti-GAPDH (14C10, Cell Signaling). The secondary antibodies used were donkey- $\alpha$ -rabbit-HRP and rabbit- $\alpha$ -goat-HRP.

## Statistical Analysis

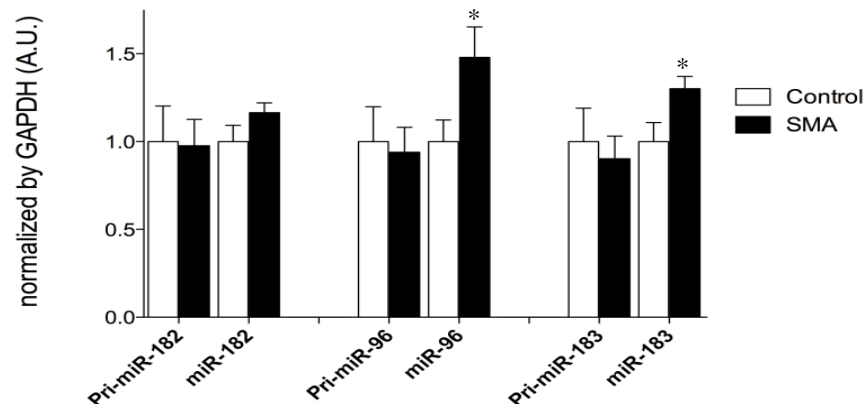
All image data was collected and analyzed using ImageJ (National Institutes of Health) and Quantity One 4.5.1 (densitometric analysis – Biorad). Statistical analysis was done with Microsoft Excel. The statistical significance was achieved applying the student's *t*-test. ( $p < 0.05 = *$ ,  $p < 0.01 = **$ , and  $p < 0.001 = ***$ ).

## RESULTS

### Quantification pri-miRNA and miRNA expression of miR-183~96~182 cluster

Our previous study showed that miR-183 expression is increased in SMN deficient cells, including spinal cord of SMA mouse model and SMN knock down cortical neuron cultures.<sup>56</sup> To understand the mechanism how SMN deficiency causes the elevated miR-183 expression, we measured the primary transcript of miR-183 in SMN deficient cells. Since miR-183 is transcribed as a single transcript from miR-183~96~182 cluster, we measured the expression of all three mature miRNAs as well as primary transcripts in 4 days old SMA mouse model (FVB, *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>). Interestingly, while the expression of miR-183 and miR-96 were elevated in spinal cord of SMA mouse, the expression of miR-182 and primary transcripts did not show any significant changes (**Figure1**).

This data suggest that there is a differential regulation in the biogenesis process among miRNA in the miR-183~96~182 cluster. Likewise, these results suggest that SMN deficiency causes dysregulation of miRNA expression, affecting the biogenesis pathway rather than general transcription.

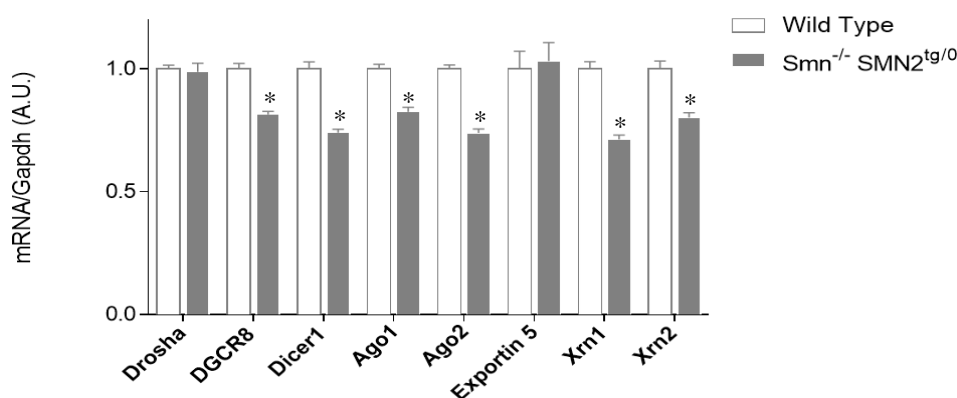


**Figure1.** The expression of miR-183~96~182 cluster in spinal cords from SMA mouse model (FVB; *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>) and control (FVB; *Smn*<sup>+/+</sup>; *SMN2*<sup>tg/0</sup>). In this experiment were used n=12 for SMA and n=8 for control. Primary transcript does not show expression changes in any of the miRNAs. Expression of mature miR-182 does not show any difference, while expression of mature miR-96 and miR-183 have significantly increased. Statistical significance was determined by Student's *t*-test: \*, *p*<0.05; \*\*, *p*<0.01; \*\*\*, *p*<0.001.

### Quantitative real time-PCR analysis of genes involved in miRNA biogenesis and decay pathways

After confirming that miR-183 expression is upregulated in spinal cords from SMA mouse model (FVB, *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>), we asked how SMN deficiency caused dysregulation in miRNA expression. We postulated that SMN protein is involved in miRNA biogenesis or stability. To test this hypothesis, we characterized miRNA biogenesis pathway from primary transcription to down-stream steps of RNA processing in spinal cords from SMA mouse model. Therefore, we designed gene-specific primers for each gene involved in biogenesis and decay pathways of miRNAs and performed a RT-PCR to measure its transcript expression levels in spinal cord.

Strikingly, mRNA expression of the many genes involved in the miRNA biogenesis pathway was significantly decreased in spinal cord of SMA mouse model. Only the expression of Drosha and Exportin5 was not altered (**Figure2**). Likewise, we observed a similar substantial reduction in the expression levels of Xrn1 and Xrn2, the exoribonucleases responsible for miRNA decay (**Figure2**).



**Figure2.** Graph shows the expression analysis of genes involved in miRNA biogenesis and decay pathways in SMA mouse model (FVB; *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>) and wild type (FVB; *Smn*<sup>+/+</sup>; *SMN2*<sup>tg/0</sup>). The error bars indicate the standard deviation (SD). Drosha and Exportin5 do not show any changes in expression, while a significant decrease is observed in the remaining genes. RT-PCR results show a dysregulated expression of genes responsible for miRNA decay and stability. Data are represented by mean  $\pm$  SD. N= 14 for SMA and n=10 for WT, except for Exportin5 with n=10 for SMA and WT (Student's *t*-test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ).

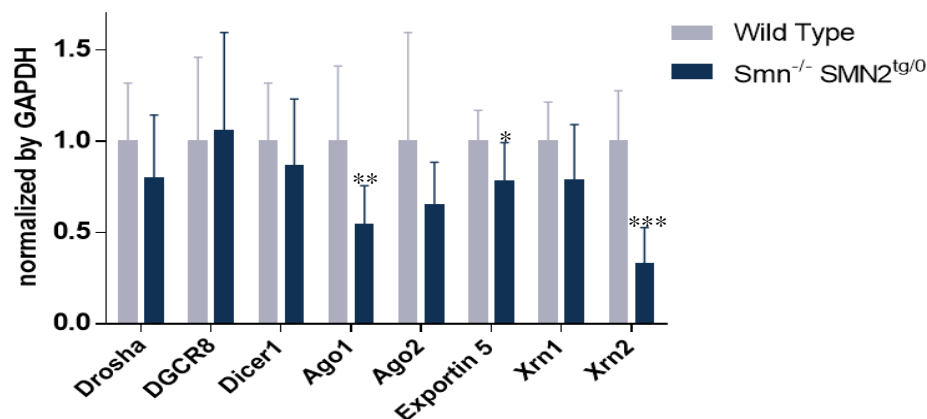
Taken together, these results showed that SMN deficiency changes the expression of the genes involved in biogenesis and stability of miRNAs. Accordingly, the observed downregulation intensely suggests that the SMN complex plays a role in miRNA expression through differential regulation of its processing and stability.

### **Characterization of splicing in genes involved in miRNA biogenesis and decay pathways**

The most well characterized function of the SMN complex is its important role in the biogenesis of U-snRNPs complexes in the nucleus.<sup>26</sup> These RNA-protein complexes are the major components of the spliceosome, where its main function is the recognition and removal of introns from pre-mRNA in the nucleus.<sup>65</sup> Since SMN protein is strongly associated with splicing, we aimed to determine if SMN causes effects in splicing process of the genes involved in miRNA biogenesis, decay and stability pathways. Specifically, we intended to uncover whether SMN deficiency leads to a disruption of the gene transcript forms, or creates new functional/nonfunctional splicing variants. To test our hypothesis, we accessed to EURASNET and collected the available information about the alternative splicing events and transcripts of genes under study. Thereafter, according to this information and the genomic structure, we design gene-specific primers flanking the exons considered as likely to undergo splicing, and performed a PCR.

Curiously, we observed that the splicing process was not dysregulated in the genes under test (**Supplementary Figure1A-H**). The amplified PCR-fragments were confirmed with Sanger sequencing. Although we did not find changes in the splicing process of these genes, we could detect significantly lower expression of some genes such as Ago1, Exportin5 and Xrn2 in SMA spinal cord (**Figure3**).





**Figure3.** Expression of genes involved in miRNA biogenesis and decay in spinal cords from SMA mouse model (FVB; *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>) and wild type (FVB; *Smn*<sup>+/+</sup>; *SMN2*<sup>tg/0</sup>). In this experiment were used n=9 for SMA and n=7 for WT, with exception of Drosha. For Drosha we used n=14 for SMA and n=14 for WT. The error bars indicate the SD. Significance was determined using the student's t-test: \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001.

This data showed that SMN does not disrupt the splicing process of the genes involved in miRNA biogenesis and decay. However, the decreased expression of some genes confirms that SMN deficiency affects the genes involved in miRNA biogenesis and decay, supporting the hypothesis that SMN is directly involved in the regulation of these pathways.

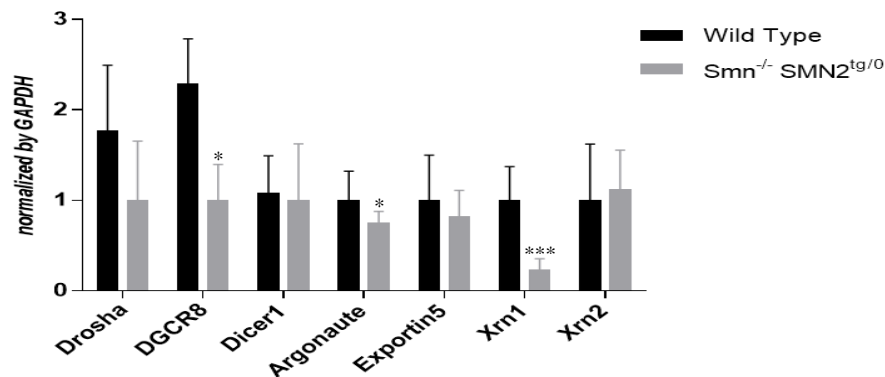
### Analysis of gene expression involved in miRNA biogenesis and decay pathways in protein level

Having demonstrated a downregulation of the genes involved in miRNA biogenesis and decay in the spinal cord of SMA mouse model, we decided to check the expression of these genes at protein level. Therefore we measured the protein expression levels of DROSHA, DGCR8, DICER, EXPORTIN5, AGO family, XRN1 and XRN2 in spinal cord of the SMA mouse model (FVB, P4, *Smn*<sup>-/-</sup>; *SMN2*<sup>tg/0</sup>) and wild type control using Western blots.

As similar to mRNA expression the protein levels of some genes such as DGCR8, AGO and XRN1 were lower in spinal cord of SMA mice compared to the wild type control (**Figure4 and Supplementary Figure2**). Among the biogenesis genes, while Drosha did not show any

alteration in its protein expression, the levels of the remaining proteins were slightly reduced in SMA samples (**Supplementary Figure 2A-E**). Specifically, the expression of DGCR8 and AGO proteins were significantly reduced (**Figure 4**). Moreover, we found a highly significant decrease in the expression of XRN1, whereas expression of XRN2 was not changed by SMN deficiency (**Figure 4 and Supplementary Figure 2F-G**).

These findings confirm our hypothesis by showing that SMN deficiency decreases the expression of the genes involved in miRNA biogenesis and decay pathways at protein level. Consequently, such results support that SMN protein regulates miRNA expression via controlling their biogenesis and decay pathways.



**Figure 4.** Quantification analysis of the protein expression levels of the genes involved in miRNA biogenesis and decay in SMA spinal cord. The error bars indicate SD. Significance was determined using the student's *t*-test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . DROSHA:  $n=6$  for SMA and  $n=8$  for WT; DGCR8:  $n=3$  for SMA and  $n=3$  for WT; DICER1:  $n=7$  for SMA and  $n=8$  for WT; AGO:  $n=12$  for SMA and  $n=12$  for WT; EXPORTIN5:  $n=8$  for SMA and  $n=7$  for WT; XRN1:  $n=9$  for SMA and  $n=6$  for WT; XRN2:  $n=7$  for SMA and  $n=5$  for WT. Results from two independent experiments. GAPDH was used as

## DISCUSSION

SMA is a devastating and lethal neuromuscular disease characterized by dysfunction/loss of alpha lower motor neurons in the anterior horns of the spinal cord.<sup>1</sup> Motor neuron degeneration has been well documented in SMA. Recently, dysregulation of miRNAs such as miR-9 and miR-9\* was reported in embryonic stem cells derived motor neurons harboring a mutation causing SMA.<sup>56</sup> The goals of this study were (1) to uncover how SMN deficiency causes increased expression of miR-183 (2) to characterize miRNA biogenesis from primary transcription to down-stream steps of RNA processing in spinal cord of a SMA mouse model.

In this study, we reported dysregulated expression of miR-183~96~182 cluster in spinal cord of SMA mouse model. The predominant mechanism for miR-183 dysregulation seems to be post-transcriptional since the transcription of miR-183 cluster is not changed by SMN deficiency. However, the molecular mechanism underlying the role of SMN protein in the dysregulation of miRNA expression remains to be elucidated.

The incorrect splicing of genes in miRNA biogenesis pathway could explain the aberrant miRNA expression. The major role of SMN protein in splicing and its involvement in the formation of RNPs complex raised the question whether its deficiency could have an impact on the splicing process and machinery.<sup>24,74</sup> To test this hypothesis, we verified a downregulation in the expression of the genes involved in miRNA biogenesis and decay in SMN deficient cells; however its splicing process was not affected. Recently, it was reported an involvement of the SMN complex in the mRNA trafficking and delivery to the axonal compartment for axonal local translation.<sup>32</sup> Thus, it is suggested that the SMN complex may play a role in the formation or trafficking of miRNA-RISC complexes and/or translational machinery. Taken together, we conclude that the SMN deficiency causes dysregulation of the miRNA expression through regulating the genes involved in miRNA related pathways.

Whichever mechanisms are contributing to the dysregulation of miRNA expression, it appears that miRNAs are differentially regulated via their biogenesis and stability pathways. In this study

study we demonstrated that SMN deficiency altered miRNA expression through a dysregulation of the genes involved in miRNA biogenesis and decay pathways.

As new strategies to uncover the molecular mechanisms underlying this dysregulation we suggest a promoter analysis of the genes involved in biogenesis and decay in SMN deficient cells. Additionally, we will investigate whether neuronal localization of these genes is affected by SMN loss.

Our study provides further knowledge about the mechanistic relationship between the SMN protein and miRNAs, which helps us to understand the role of miRNA pathway underlying SMA pathology.

## ACKNOWLEDGEMENTS

I would like to thank to all those without whom I would not have been able to complete this dissertation. First and foremost, I would like to express my deepest gratitude to my supervisor Min Joung Kye, whose guidance, assistance and encouragement have been crucial to the materialization of my research into this Master dissertation. For the opportunity to work on this project, for everything she taught me and for all the help in writing this manuscript. I would like also to thank Prof. Dr. Júlio António Bargão Duarte for all the help provided for the completion of this dissertation. I also thank Prof. Dr. Manuel Carmo Gomes, the Master coordinator, for all the assistance provided. My sincere gratitude goes for Prof. Dr. Brunhilde Wirth, the director of the Institute for Human Genetics, for supporting the realization of this work by giving me the opportunity to work with Dr. Min Jeong Kye and perform my research in her laboratory. I'm grateful to all the members of Prof. Dr. Brunhilde group, for all the patience and caring while guiding and always help when needed in the laboratory.

I thank all the members of my family for all the support and encouragement. My greatest gratitude goes to my parents, to whom this dissertation is dedicated to, for the unconditional support, both financial and emotionally, love, patience and encouragement. I also thank my godparents for always treating me like a daughter, giving me so much love, encouragement and being always so caring. Additionally, I thank my friends, Maria, Filipa, Denise, Giovanna, Lúcia, Lucía, Márcia, Ana Rita, Brian, Margarida, Toga, Pedro, Gonçalo Matos, Gonçalo Hilário, Pedro Chambel, Rita, Joana, Vítor, Mauro, Maria Rita, Tânia, Mafalda, Bárbara, Ricardo, Nuno, Mínia, Duarte, David e Miguel, for being always with me, offering me so much happy moments, giving me support and being so caring. My special thanks goes to my closest friends, Maria, Filipa, for all the support while I was in Germany developing my dissertation. I am very thankful to Filipa for being so kind and help me with everything in Portugal. Finally, I would like to thank my boyfriend, Levin Melches, for the constant support and encouragement, caring and patience, being with me through the good and bad times.

## REFERENCES

- 1 Crawford, T. O. & Pardo, C. A. The neurobiology of childhood spinal muscular atrophy. *Neurobiology of disease* **3**, 97-110, doi:10.1006/nbdi.1996.0010 (1996).
- 2 Wirth, B., Brichta, L. & Hahnen, E. Spinal muscular atrophy: from gene to therapy. *Seminars in pediatric neurology* **13**, 121-131, doi:10.1016/j.spen.2006.06.008 (2006).
- 3 Pearn, J. Classification of spinal muscular atrophies. *Lancet* **1**, 919-922 (1980).
- 4 Markowitz, J. A., Tinkle, M. B. & Fischbeck, K. H. Spinal muscular atrophy in the neonate. *Journal of obstetric, gynecologic, and neonatal nursing : JOGNN / NAACOG* **33**, 12-20 (2004).
- 5 Munsat, T. L. & Davies, K. E. International SMA consortium meeting. (26-28 June 1992, Bonn, Germany). *Neuromuscular disorders : NMD* **2**, 423-428 (1992).
- 6 Russman, B. S. Spinal muscular atrophy: clinical classification and disease heterogeneity. *Journal of child neurology* **22**, 946-951, doi:10.1177/0883073807305673 (2007).
- 7 Lunn, M. R. & Wang, C. H. Spinal muscular atrophy. *Lancet* **371**, 2120-2133, doi:10.1016/S0140-6736(08)60921-6 (2008).
- 8 Zerres, K., Wirth, B. & Rudnik-Schoneborn, S. Spinal muscular atrophy--clinical and genetic correlations. *Neuromuscular disorders : NMD* **7**, 202-207 (1997).
- 9 Lefebvre, S. *et al.* Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **80**, 155-165 (1995).
- 10 Brzustowicz, L. M. *et al.* Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. *Nature* **344**, 540-541, doi:10.1038/344540a0 (1990).
- 11 Burghes, A. H. & Beattie, C. E. Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? *Nature reviews. Neuroscience* **10**, 597-609, doi:10.1038/nrn2670 (2009).
- 12 Lorson, C. L., Hahnen, E., Androphy, E. J. & Wirth, B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 6307-6311 (1999).
- 13 Burglen, L. *et al.* Structure and organization of the human survival motor neurone (SMN) gene. *Genomics* **32**, 479-482, doi:10.1006/geno.1996.0147 (1996).
- 14 Monani, U. R. *et al.* A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Human molecular genetics* **8**, 1177-1183 (1999).
- 15 Shababi, M., Lorson, C. L. & Rudnik-Schoneborn, S. S. Spinal muscular atrophy: a motor neuron disorder or a multi-organ disease? *Journal of anatomy*, doi:10.1111/joa.12083 (2013).
- 16 Feldkotter, M., Schwarzer, V., Wirth, R., Wienker, T. F. & Wirth, B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *American journal of human genetics* **70**, 358-368, doi:10.1086/338627 (2002).
- 17 Lefebvre, S. *et al.* Correlation between severity and SMN protein level in spinal muscular atrophy. *Nature genetics* **16**, 265-269, doi:10.1038/ng0797-265 (1997).
- 18 Wirth, B. *et al.* Mildly affected patients with spinal muscular atrophy are partially protected by an increased SMN2 copy number. *Human genetics* **119**, 422-428, doi:10.1007/s00439-006-0156-7 (2006).
- 19 Coovert, D. D. *et al.* The survival motor neuron protein in spinal muscular atrophy. *Human molecular genetics* **6**, 1205-1214 (1997).
- 20 Schrank, B. *et al.* Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 9920-9925 (1997).

- 21 Liu, Q. & Dreyfuss, G. A novel nuclear structure containing the survival of motor neurons protein. *The EMBO journal* **15**, 3555-3565 (1996).
- 22 Setola, V. *et al.* Axonal-SMN (a-SMN), a protein isoform of the survival motor neuron gene, is specifically involved in axonogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 1959-1964, doi:10.1073/pnas.0610660104 (2007).
- 23 Pellizzoni, L. Chaperoning ribonucleoprotein biogenesis in health and disease. *EMBO reports* **8**, 340-345, doi:10.1038/sj.embor.7400941 (2007).
- 24 Carissimi, C. *et al.* Gemin8 is a novel component of the survival motor neuron complex and functions in small nuclear ribonucleoprotein assembly. *The Journal of biological chemistry* **281**, 8126-8134, doi:10.1074/jbc.M512243200 (2006).
- 25 Carissimi, C., Saieva, L., Gabanella, F. & Pellizzoni, L. Gemin8 is required for the architecture and function of the survival motor neuron complex. *The Journal of biological chemistry* **281**, 37009-37016, doi:10.1074/jbc.M607505200 (2006).
- 26 Pellizzoni, L., Yong, J. & Dreyfuss, G. Essential role for the SMN complex in the specificity of snRNP assembly. *Science* **298**, 1775-1779, doi:10.1126/science.1074962 (2002).
- 27 Winkler, C. *et al.* Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. *Genes & development* **19**, 2320-2330, doi:10.1101/gad.342005 (2005).
- 28 Bechade, C. *et al.* Subcellular distribution of survival motor neuron (SMN) protein: possible involvement in nucleocytoplasmic and dendritic transport. *The European journal of neuroscience* **11**, 293-304 (1999).
- 29 Pagliardini, S. *et al.* Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Human molecular genetics* **9**, 47-56 (2000).
- 30 Zhang, H. L. *et al.* Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**, 6627-6637 (2003).
- 31 Zhang, H. *et al.* Multiprotein complexes of the survival of motor neuron protein SMN with Gemin traffic to neuronal processes and growth cones of motor neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**, 8622-8632, doi:10.1523/JNEUROSCI.3967-05.2006 (2006).
- 32 Akten, B. *et al.* Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 10337-10342, doi:10.1073/pnas.1104928108 (2011).
- 33 Rossoll, W. *et al.* Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *The Journal of cell biology* **163**, 801-812, doi:10.1083/jcb.200304128 (2003).
- 34 Sharma, A. *et al.* A role for complexes of survival of motor neurons (SMN) protein with gemins and profilin in neurite-like cytoplasmic extensions of cultured nerve cells. *Experimental cell research* **309**, 185-197, doi:10.1016/j.yexcr.2005.05.014 (2005).
- 35 McWhorter, M. L., Monani, U. R., Burghes, A. H. & Beattie, C. E. Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *The Journal of cell biology* **162**, 919-931, doi:10.1083/jcb.200303168 (2003).
- 36 Kariya, S. *et al.* Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Human molecular genetics* **17**, 2552-2569, doi:10.1093/hmg/ddn156 (2008).
- 37 Kong, L. *et al.* Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 842-851, doi:10.1523/JNEUROSCI.4434-08.2009 (2009).
- 38 Yamazaki, T. *et al.* FUS-SMN protein interactions link the motor neuron diseases ALS and SMA. *Cell reports* **2**, 799-806, doi:10.1016/j.celrep.2012.08.025 (2012).

- 39 Piazzon, N. *et al.* In vitro and in cellulo evidences for association of the survival of motor neuron complex with the fragile X mental retardation protein. *The Journal of biological chemistry* **283**, 5598-5610, doi:10.1074/jbc.M707304200 (2008).
- 40 Tadesse, H., Deschenes-Furry, J., Boisvenue, S. & Cote, J. KH-type splicing regulatory protein interacts with survival motor neuron protein and is misregulated in spinal muscular atrophy. *Human molecular genetics* **17**, 506-524, doi:10.1093/hmg/ddm327 (2008).
- 41 Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233, doi:10.1016/j.cell.2009.01.002 (2009).
- 42 Krol, J., Loedige, I. & Filipowicz, W. The widespread regulation of microRNA biogenesis, function and decay. *Nature reviews. Genetics* **11**, 597-610, doi:10.1038/nrg2843 (2010).
- 43 Kim, V. N. MicroRNA biogenesis: coordinated cropping and dicing. *Nature reviews. Molecular cell biology* **6**, 376-385, doi:10.1038/nrm1644 (2005).
- 44 Macias, S., Cordiner, R. A. & Caceres, J. F. Cellular functions of the microprocessor. *Biochemical Society transactions* **41**, 838-843, doi:10.1042/BST20130011 (2013).
- 45 Yi, R., Qin, Y., Macara, I. G. & Cullen, B. R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development* **17**, 3011-3016, doi:10.1101/gad.1158803 (2003).
- 46 Chendrimada, T. P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740-744, doi:10.1038/nature03868 (2005).
- 47 Lee, Y. *et al.* The role of PACT in the RNA silencing pathway. *The EMBO journal* **25**, 522-532, doi:10.1038/sj.emboj.7600942 (2006).
- 48 MacRae, I. J., Ma, E., Zhou, M., Robinson, C. V. & Doudna, J. A. In vitro reconstitution of the human RISC-loading complex. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 512-517, doi:10.1073/pnas.0710869105 (2008).
- 49 Kim, V. N., Han, J. & Siomi, M. C. Biogenesis of small RNAs in animals. *Nature reviews. Molecular cell biology* **10**, 126-139, doi:10.1038/nrm2632 (2009).
- 50 Gregory, R. I., Chendrimada, T. P., Cooch, N. & Shiekhattar, R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123**, 631-640, doi:10.1016/j.cell.2005.10.022 (2005).
- 51 Diederichs, S. & Haber, D. A. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell* **131**, 1097-1108, doi:10.1016/j.cell.2007.10.032 (2007).
- 52 Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nature cell biology* **11**, 228-234, doi:10.1038/ncb0309-228 (2009).
- 53 Kai, Z. S. & Pasquinelli, A. E. MicroRNA assassins: factors that regulate the disappearance of miRNAs. *Nature structural & molecular biology* **17**, 5-10, doi:10.1038/nsmb.1762 (2010).
- 54 Ramachandran, V. & Chen, X. Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. *Science* **321**, 1490-1492, doi:10.1126/science.1163728 (2008).
- 55 Kosik, K. S. The neuronal microRNA system. *Nature reviews. Neuroscience* **7**, 911-920, doi:10.1038/nrn2037 (2006).
- 56 Kye, M. J. *et al.* Somatodendritic microRNAs identified by laser capture and multiplex RT-PCR. *RNA* **13**, 1224-1234, doi:10.1261/rna.480407 (2007).
- 57 Schratt, G. M. *et al.* A brain-specific microRNA regulates dendritic spine development. *Nature* **439**, 283-289, doi:10.1038/nature04367 (2006).
- 58 Banerjee, S., Neveu, P. & Kosik, K. S. A coordinated local translational control point at the synapse involving relief from silencing and MOV10 degradation. *Neuron* **64**, 871-884, doi:10.1016/j.neuron.2009.11.023 (2009).
- 59 Mourelatos, Z. *et al.* miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes & development* **16**, 720-728, doi:10.1101/gad.974702 (2002).



- 60 Dostie, J., Mourelatos, Z., Yang, M., Sharma, A. & Dreyfuss, G. Numerous microRNPs in neuronal cells containing novel microRNAs. *RNA* **9**, 180-186 (2003).
- 61 Haramati, S. *et al.* miRNA malfunction causes spinal motor neuron disease. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 13111-13116, doi:10.1073/pnas.1006151107 (2010).
- 62 Riessland, M. *et al.* SAHA ameliorates the SMA phenotype in two mouse models for spinal muscular atrophy. *Human molecular genetics* **19**, 1492-1506, doi:10.1093/hmg/ddq023 (2010).
- 63 Kye, M. J. *et al.* NMDA mediated contextual conditioning changes miRNA expression. *PloS one* **6**, e24682, doi:10.1371/journal.pone.0024682 (2011).
- 64 Sambrook, J. & Russell, D. W. *The condensed protocols from Molecular cloning : a laboratory manual*. (Cold Spring Harbor Laboratory Press, 2006).
- 65 Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. Are snRNPs involved in splicing? *Nature* **283**, 220-224 (1980).
- 66 Lee, S. T. *et al.* miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model. *Annals of neurology* **72**, 269-277, doi:10.1002/ana.23588 (2012).
- 67 Gaughwin, P. M. *et al.* Hsa-miR-34b is a plasma-stable microRNA that is elevated in pre-manifest Huntington's disease. *Human molecular genetics* **20**, 2225-2237, doi:10.1093/hmg/ddr111 (2011).
- 68 Abelson, J. F. *et al.* Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science* **310**, 317-320, doi:10.1126/science.1116502 (2005).
- 69 Williams, A. H. *et al.* MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science* **326**, 1549-1554, doi:10.1126/science.1181046 (2009).
- 70 Li, J. *et al.* miR-183 inhibits TGF-beta1-induced apoptosis by downregulation of PDCD4 expression in human hepatocellular carcinoma cells. *BMC cancer* **10**, 354, doi:10.1186/1471-2407-10-354 (2010).
- 71 Mihelich, B. L. *et al.* miR-183-96-182 cluster is overexpressed in prostate tissue and regulates zinc homeostasis in prostate cells. *The Journal of biological chemistry* **286**, 44503-44511, doi:10.1074/jbc.M111.262915 (2011).
- 72 Zhu, W. *et al.* Overexpression of members of the microRNA-183 family is a risk factor for lung cancer: a case control study. *BMC cancer* **11**, 393, doi:10.1186/1471-2407-11-393 (2011).
- 73 Zhao, H. *et al.* miR-183 inhibits the metastasis of osteosarcoma via downregulation of the expression of Ezrin in F5M2 cells. *International journal of molecular medicine* **30**, 1013-1020, doi:10.3892/ijmm.2012.1111 (2012).
- 74 Mourelatos, Z., Abel, L., Yong, J., Kataoka, N. & Dreyfuss, G. SMN interacts with a novel family of hnRNP and spliceosomal proteins. *The EMBO journal* **20**, 5443-5452, doi:10.1093/emboj/20.19.5443 (2001).
- 75 Hu, Y. H. *et al.* Cell array-based intracellular localization screening reveals novel functional features of human chromosome 21 proteins. *BMC genomics* **7**, 155, doi:10.1186/1471-2164-7-155 (2006).

# The role of SMN protein in microRNA biogenesis



**INSTITUTE OF  
HUMAN GENETICS**  
UNIVERSITY OF COLOGNE

Inês do Carmo Gil Gonçalves

Supervised by:

Min Jeong Kye, PhD

Júlio António Bargão Duarte, PhD

## **Supplemental Data**

M. Sc. in Molecular Biology and Genetics

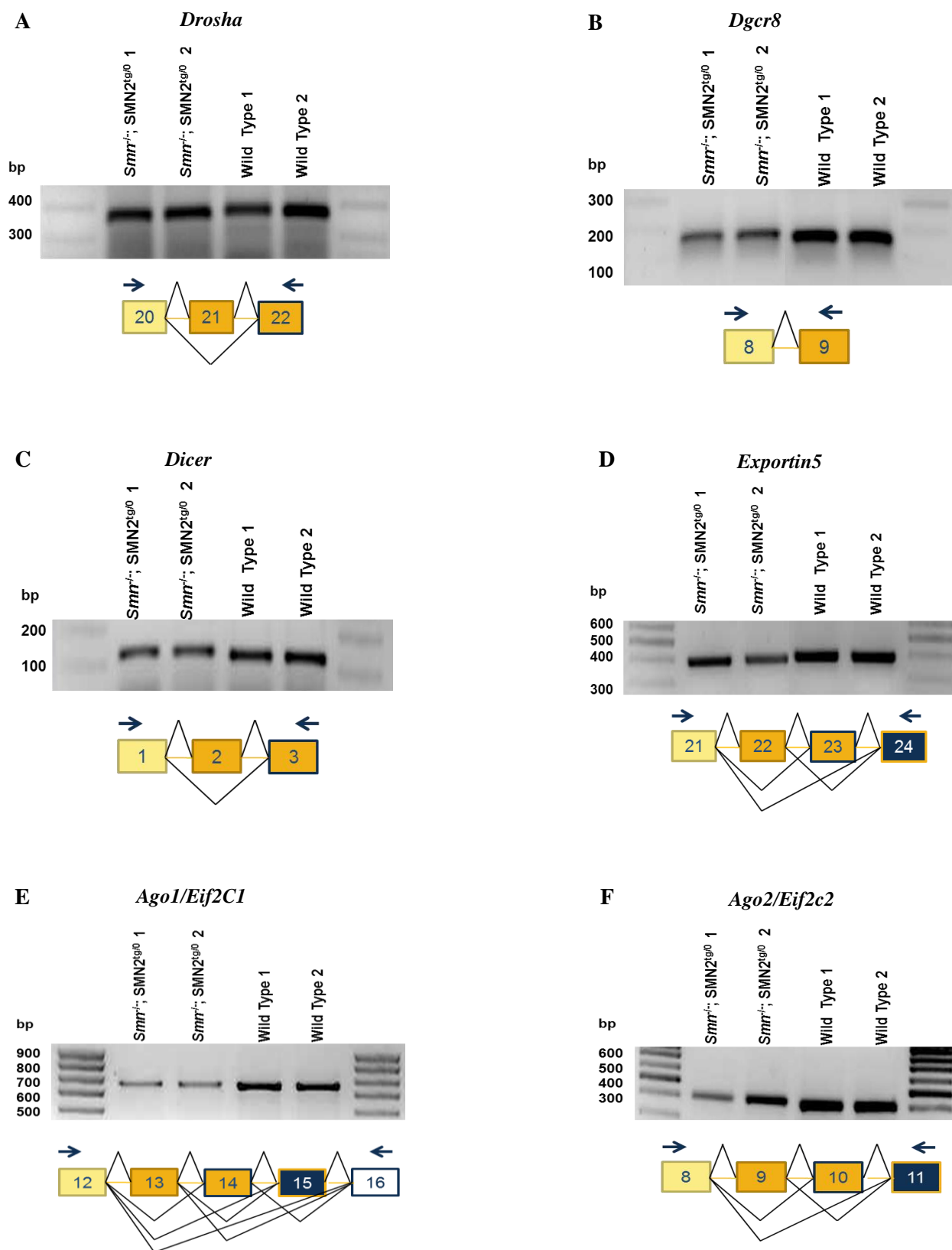
2013

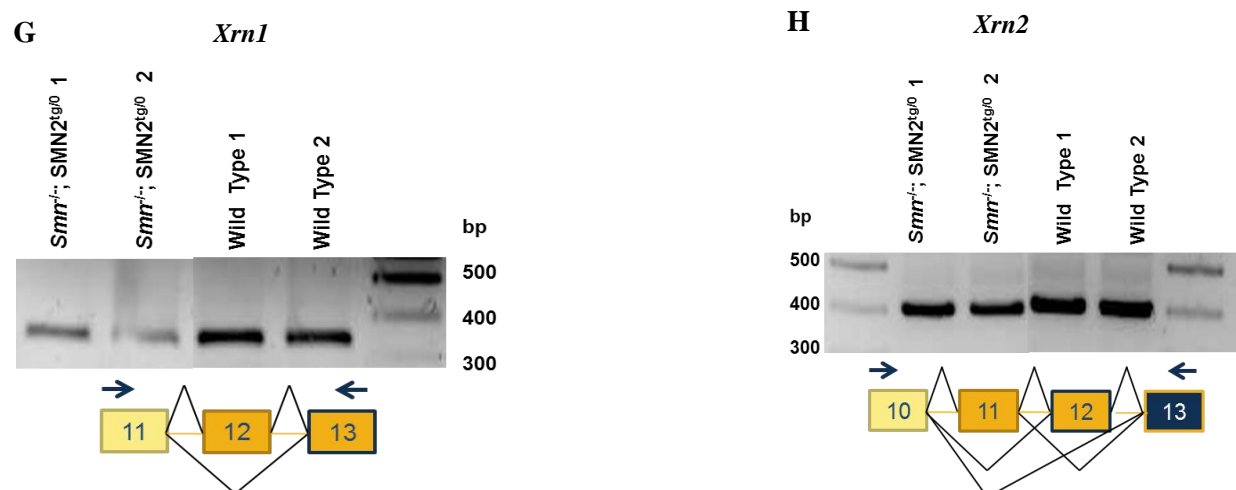
**Supplementary Table1.** Primer sequences to measure the mRNA expression of the genes involved in miRNA biogenesis and stability in spinal cord from SMA mouse model.

Gene	5' → 3' forward primer	5' → 3' reverse primer	Annealing/ Amplicon length (bp)
mDrosha	GGACCATCACGAAGGACACT	GATGTACAGCGCTGCGATAA	55°C/197
mDGCR8	GAAACCATGGAATGGGTGAC	CAGAGGTCTCCTGCTTGACC	58°C/250
mDicer	GTGGAGGGAGACCAGTCAAA	TGGGAAGCTATGGGTTCTTG	55°C/250
mEif2C1	TCGGAAGATTTCCAAGGATG	GTTGCCATTCCCAAGAGTGT	53°C/216
mEIF2C2	AAGTCGGACAGGAGCAGAAA	GAAACTTGCACTTCGCATCA	52°C/182
mXPO5	TTCCTGACTTCCGGCTTAGA	TGGTTGATGACATGCCACTT	55°C/160
mXRN1	GAGATGAGCGTGGAGTGTCA	CGCAGAAAGGAGAAATCAGG	55°C/230
mXRN2	TTGAGAAGCAGCGAGTCAGA	CCAGGTGCACTAGCATCAGA	53°C/217
mGAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA	60°C

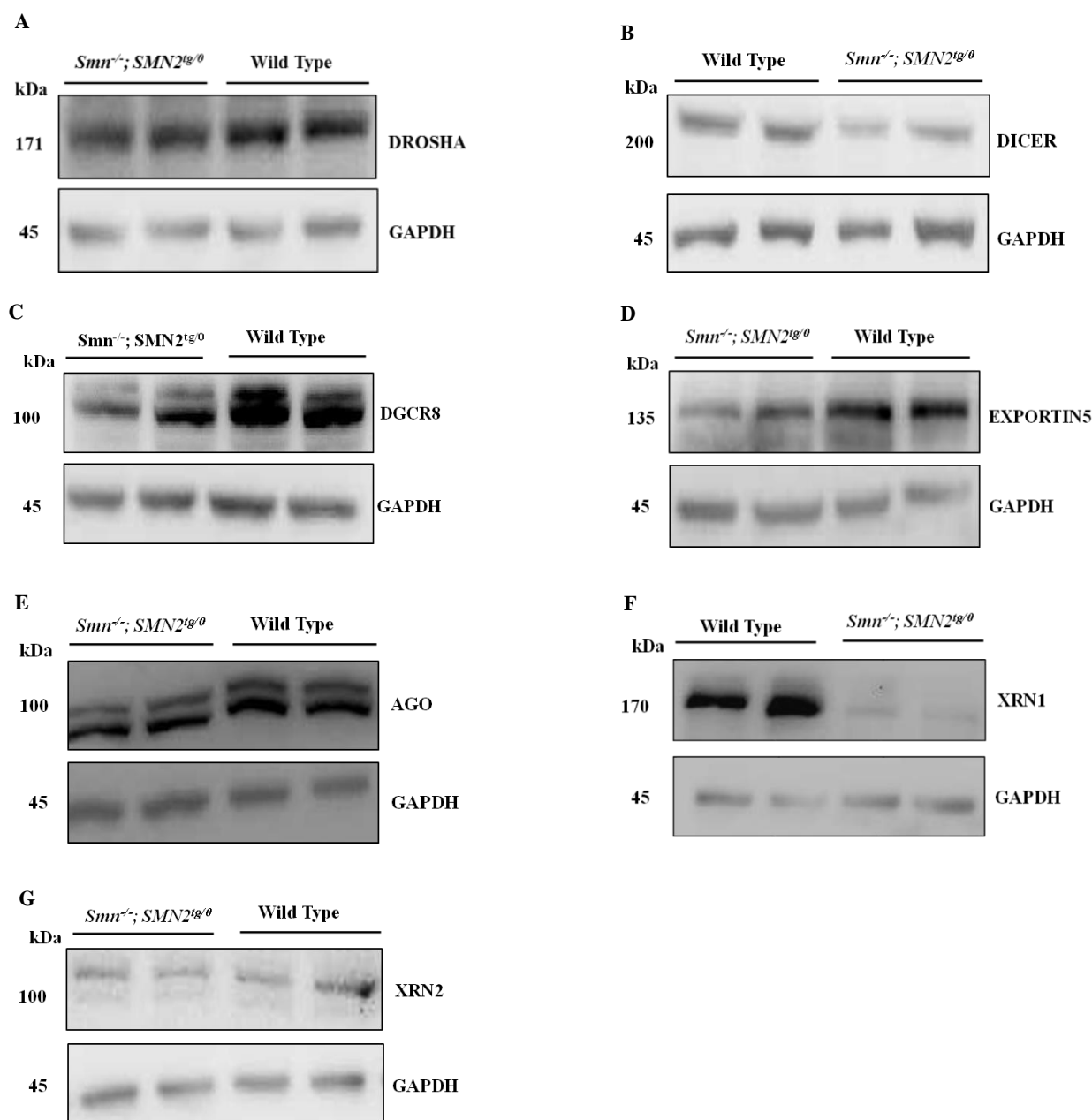
**Supplementary Table2.** Primer sequences to study the splicing of the genes involved in miRNA biogenesis and stability in spinal cord of SMA mouse model.

Gene	5' → 3' forward primer	5' → 3' reverse primer	Annealing/ Amplicon length (bp)
mDrosha	CATCACATCCGGTACCATCA	GAAGGAGTTGGATCATCTTGG	53°C/308
mDGCR8	AATCCAAGTGAGCCTTTTGGT	GCTTCTCCTCAGAGGTCTGTTT	60°C/154
mDicer	TGGGTCCTTTCTTTGGACTG	GAACACGGTCCTTTTTCAT	58°C/183
mEif2C1-a	TCGGAAGATTTCCAAGGATG	CGCTCATTCTTGTACGACA	55°C/747
mEif2C1-b	CTGCCATGTGGAAGATGATG	ACTTCCACCTTCAGGCCTTT	58°C/184
mEIF2C2-a	CGCGTCGGGTAAACCTGT	GATGCGATCTTTGCCTTCTC	53°C/522
mEIF2C2-b	AAGTCGGACAGGAGCAGAAA	TGAGATGGACTTCTGTACACTGG	53°C/417
mXPO5	ACCGGAAATGCTAACGAAAA	GAGGTCCAAGGATGGGAGAT	58°C/420
mXRN1	TTGGGCTGCATTAGACAAAA	CCAGGACTGGACTCCATGAT	55°C/242
mXRN2	TCTT CCTTCGGCTG AATGTC	ATCCTCAACTTCACCAACTGC	53°C/374





**Supplementary Figure1.** Schematic representation of PCR results from the analysis of alternative splicing of genes involved in miRNA biogenesis and decay pathways. Exons are represented by boxes filled with the respective exon number, introns by yellow lines, patterns of alternative splicing by thin black lines, and arrows indicated the primer-pairs position. Exon skipping was not observed in any of the genes in study. Molecular weight marker size is indicated. (A) *Drosha*. Position of predicted alternative spliced products with exon 21, 22 and 23 inclusion: 360 bp. (B) *Dgcr8*. Primer extension product predicted size is 170 bp (inclusion of exons 8 and 9). (C) *Dicer*. Amplified products have an expected size of 183bp for the inclusion of exons 8 and 9). (D) *Exportin5*. Primer extension product predicted for intron 21, 22, 23 and 24 inclusion. (E) *Ago1/Eif2c1*. Amplified fragments with a predicted size of 720 bp (inclusion of exon 12, 13, 14, 15 and 16) (F) *Ago2/Eif2c2*. Position of predicted alternative spliced products with exon 8, 9, 10 and 11 inclusion: 417 bp. (G) *Xrn1*. Fragments amplified by PCR have with predicted size of 250bp (inclusion of exon 11, 12, and 13). (H) *Xrn2*. Position of predicted alternative spliced products with exon 10, 11 and 12 inclusion: 380 bp.



**Supplementary Figure2.** Western blot analysis of the gene expression at protein level in spinal cord of SMA mouse model. 30µg/mL of protein lysate was loaded for all the samples tested. Primary antibodies were incubated with a 1:350 dilution factor, while secondary antibody was used in a 1:5000 dilution. The expected molecular weights are indicated in the schematic representation of each performed Western Blot. GAPDH was used as internal control to monitor loading efficiency. (A) DROSHA. (B) DICER. (C) DGCR8. (D) EXPORTIN5. (E) AGO. (F) XRN1. (G) XRN2.